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The effect of the hexahistidine-tag in the oligomerization of HSC70 constructs

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Abstract

The hexahistidine is a fusion tag used for the isolation of proteins via an immobilized metal-ion affinity chromatography (IMAC). In the present study, we have purified and analyzed two constructs of the heat shock protein HSC70 in the presence or the absence of the His-tag (C30WT-His^+/C30WT and C30ΔL-His^+/C30ΔL). The oligomerization properties of the constructs were analyzed by size exclusion chromatography (SEC) and analytical ultracentrifugation (AU). Results from SEC analysis indicated that the His-tag promotes the dimerization of C30ΔL-His^+ but has no effect on the elution profile of C30WT-His^+, compared to their respective untagged forms C30ΔL and C30WT. These observations were also confirmed by AU analysis which indicates that C30ΔL is stabilized in the dimeric form in the presence of the His-tag. These results emphasize the need to remove the His-tag before structural characterization of some recombinant proteins.

Keywords: Immobilized metal affinity chromatography (IMAC); HSC70; Mutagenesis; Histidine-tag; Oligomerization

1. Introduction

The 70 kDa heat shock proteins (HSP70) are ubiquitous proteins, present in all organisms and cell compartments, which play an important role in thermotolerance, protein folding, protein assembly and disassembly, protein transport and signal transduction [1–3]. The constitutively expressed 70 kDa heat shock cognate protein (HSC70), a member of the HSP70 proteins, binds peptides and unfolded proteins [4,5]. HSC70 is composed of an N-terminal ATPase domain (residues 1–384) and a C-terminal domain (C30WT: residues 385–646) that can be divided into a peptide binding sub-domain (residues 385–540) and a C-terminal sub-domain (residues 540–646) [6–8]. HSP70 family members from bacteria to man oligomerize into several species in vitro [9–11] and in vivo [12–14], and even though the role of self-association has not yet been established, mounting evidence suggests the existence of a relationship between oligomerization and chaperone activity. The oligomerization of the HSP70 proteins is regulated by ATP binding, co-chaperones and peptides [15–17], and the C-terminal domain of the protein (residues 385–646), which is necessary for the chaperone activity since it holds the peptide binding site, has also been involved in oligomerization [16]. Therefore, the quaternary structure of the HSP70 proteins and the structural basis of its formation need to be elucidated for a better understanding of the biological function of these proteins. To this end, we have investigated in our laboratory the structural basis of self-association of rat HSC70 proteins through the physico-chemical and structural characterization of several deleted constructs of the protein.

For large-scale purification of recombinant proteins, immobilized metal-ion affinity chromatography (IMAC) is usually preferred due to the specificity of the process and the relatively easy purification schemes [18–20]. Chimeric proteins have been made with affinity tags like β-galactosidase, *Escherichia coli* maltose binding protein, FLAG peptide, glutathione-S-transferase and hexahistidine tag (His-tag) [21]. The His-tag, consisting of four to six consecutive Histidine residues, functions as a predominant ligand in the IMAC [22]. The ability of the Histidine residue complex to bind to metal ions with high affinity even in the presence of denaturing agents and the requirement of mild elution conditions has made the His-tag a versatile tool for protein purification and characterization [23,24]. Moreover, a facile and specific detection of His-tag can be achieved by using anti-His antibodies [25]. Therefore, several cloning vectors con-

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taining a C- or N-terminal in-frame sequence for His-tag have been engineered to enable the expression of recombinant proteins in prokaryotic and eukaryotic expression systems [26–30]. Although universally applicable, the use of His-tags and IMAC purification is not recommended for proteins containing metal ions. Similarly, other amino acids like cysteine and naturally occurring histidine rich regions in host proteins may result in unwanted protein binding during IMAC purification [31]. Additionally, introducing an affinity tag has also been reported to negatively affect the target protein resulting in a change in protein conformation [32], undesired flexibility in structure studies [33], inhibition of enzyme activity, toxicity [34,35] and alteration in biological activity [36].

In the present study, we have investigated the effect of the hexahistidine purification tag on the self-association of rat HSC70 protein through the physico-chemical and structural characterization of deleted constructs of the protein. To this end, we have engineered two different constructs: the construct corresponding to the C-terminal domain (C30WT: residues 385–646) and the construct corresponding to the C-terminal domain bearing deletion of the conserved Leucine residues located in the interdomain hydrophobic linker (C30ΔL: residues 395–646 with deletion of Leu391-Leu394), with a hexahistidine purification tag attached to their NH_2-terminus and expressed in *E. coli*. The recombinant proteins were analyzed by size exclusion chromatography and analytical ultracentrifugation. The results demonstrate that although purification of His-tagged recombinant proteins by Ni^{2+} chelate affinity chromatography is rapid and efficient, the presence of the additional His-tag can significantly alter the self-association properties of some HSC70 constructs.

2. Experimental

2.1. Materials

Membranes for ultrafiltration were performed by Amicon. Restriction endonucleases and T4 DNA ligase were from New England Biolabs. pET-14b vector was purchased from TebuNovagen. Ni^{2+}-agarose was purchased from Quiagen. Liquid chromatography materials, FPLC products and columns were from Pharmacia Amersham. DNA oligonucleotides and DNA sequencing were purchased from MWG-Biotech. Electrophoresis supply was from BioRad and all other chemicals were from Merck or Sigma.

2.2. Construction of the mutant proteins

The C-terminal domain of rat HSC70 (C30WT: residues 385–646) and the construct C30ΔL (residues 395–646) are those in which the entire N-terminal domain has been deleted. C30ΔL corresponds also to the C-terminal domain of HSC70 bearing deletion of Leu391-Leu394 (see Fig. 1). Named relative to their theoretical molecular weight, proteins have been constructed using the pFB7 expression vector as described previously [16,17]. For the construction of the wild type C-terminal domain C30WT and the C-terminal domain construct C30ΔL, an NdeI restriction site has been introduced in the pFB7 plasmid using 32-mer oligonucleotides targeting a DNA region, thus replacing respectively, codon 384 and codon 394 of HSC70 by a start codon. The NdeI-BamHI fragments from each of these plasmids were subcloned into pET-14b vector, thus resulting in the C30WT and C30ΔL mutant proteins fused to a hexahistidine-tag at their NH_2-terminus.

The integrity of all constructions was verified by automatic nucleotide sequencing (MWG-Biotech).

3. Expression and purification of the mutant proteins

Proteins were expressed using BL21 pLysS *E. Coli* cells (Stratagene). BL21 pLysS *E. Coli* cells bearing plasmids expressing C30WT and C30ΔL were incubated overnight in 50 ml of LB medium containing 200 μg/ml Ampicillin at 37 °C. After dilution in the same fresh medium and growth to an OD_{600} of 0.6, expression of protein was induced by the addition of 0.5 mM of IPTG for 3 h at 37 °C. Cells were recovered after centrifugation at 10,000 rpm for 20 min at 4 °C and resuspended in binding buffer (300 mM NaCl; 50 mM Tris/HCl pH 8; 10 mM Imidazole) supplemented with 1 mM PMSF and bacteria were lysed by sonication. The lysate was centrifuged twice for 20 min at 20,000 at 4 °C to eliminate cellular debris. The supernatant was loaded into a 10 ml Ni^{2+}-agarose column pre-equilibrated with binding buffer. The column was washed with three volumes of binding buffer at flow rate of 0.2 ml/min, followed by three volumes of wash buffer (300 mM NaCl; 50 mM Tris/HCl pH 8; 20 mM Imidazole). The His-tagged protein was then eluted by a step with three volumes of elution buffer (300 mM NaCl; 50 mM Tris/HCl pH 8; 250 mM Imidazole), and fractions of 2 ml were collected. The fractions containing His-tagged proteins C30WT-His^+ and C30ΔL-His^+ were pooled. Some fractions were diluted two times for thrombin digestion, in order to obtain C30WT and C30ΔL without the His-tag. Any un-cleaved His-tagged protein was removed by resubmitting the whole sample on the Ni^{2+}-agarose column rigorously washed. After elution, the buffer was exchanged. Proteins were concentrated by successive cycles of ultra-filtration. Purified proteins were stored at −80 °C in the elution buffer described above and supplemented with 10% glycerol.

The protein concentration was determined by the Lowry method using Bovine Serum Albumin as standard, and all protein concentrations given in the figures are based on the molecular weight of the monomer.

4. Electrophoresis

Polyacrylamide gel electrophoresis (PAGE) under denaturing conditions (SDS) was carried out in 0.75 mm thick 15% acrylamide slab gels according to Laemelli method (1970) and the proteins were detected using Coomassie blue R-250. Proteins were mixed with 1/3 of the final volume of loading buffer containing 5% SDS, 30% glycerol, 3 mM DTT, and 0.01% bromophenol blue. The sample was heated at 95 °C for 10 min and loaded on a SDS–PAGE. Gels were run using the Mini-Protean II apparatus and molecular weight standards from Bio-Rad.

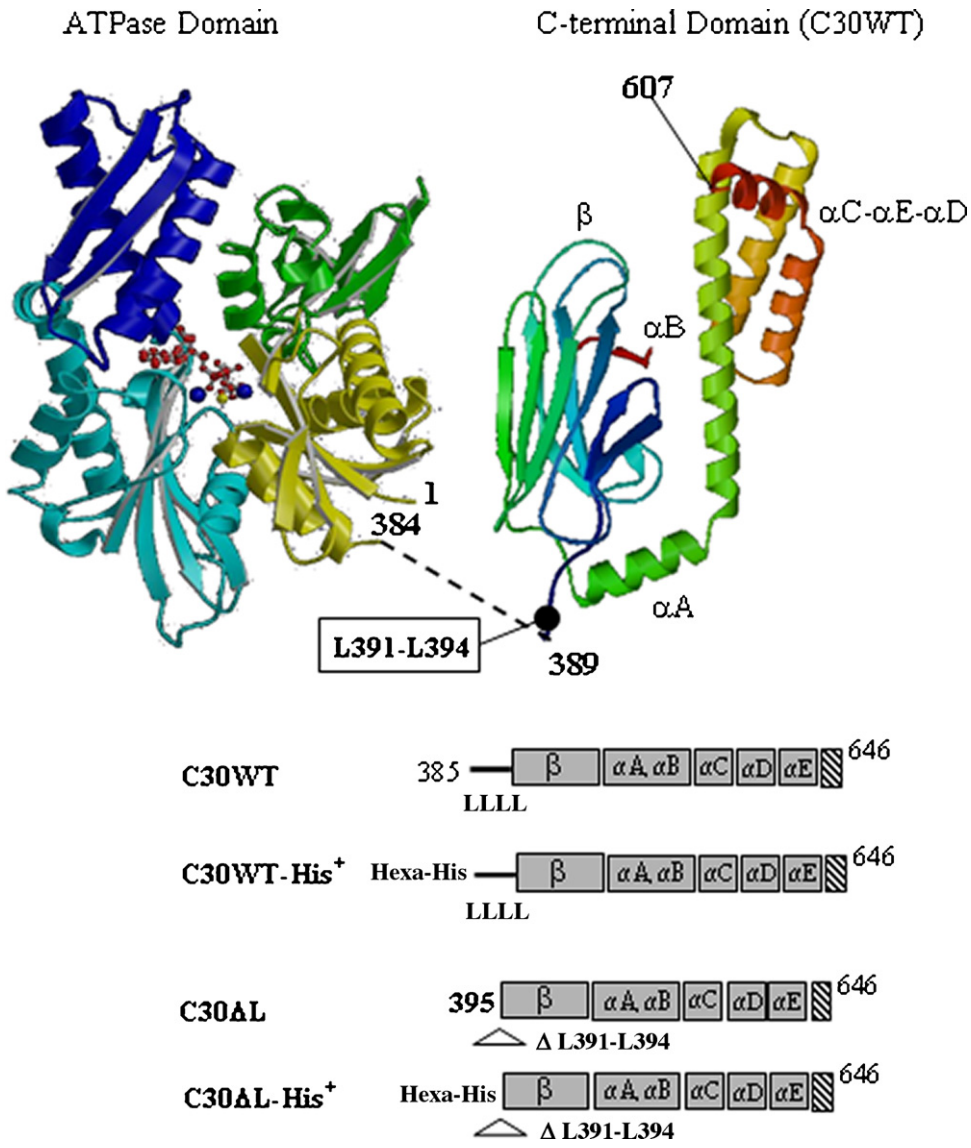

Fig. 1. Schematic representation of HSC70 mutants. *Top*: ribbon diagram of HSC70 ATPase domain (Flaherty et al., 1990; PDB entry: 3HSC) and DnaK peptide-binding domain (Zhu et al., 1996; PDB entry: 1DKZ). The position of the deletion is indicated by dots. *Bottom*: schematic outline of C30WT, C30WT-His$^+$ and the deletion constructs C30ΔL and C30ΔL-His$^+$.

5. Size-exclusion chromatography

FPLC chromatography was carried out at 20 °C on a Superdex200 (HR 10/30) column equilibrated with (20 mM Tris HCl pH 7.5; 100 mM KCl; 3 mM $MgCl_2$; 1 mM EDTA pH 8). Chromatography was performed after incubation of the proteins for 2 h at 20 °C in the buffer described above. Proteins were centrifuged 30 min at 4 °C and 10,000 rpm before application to the column. Fractions of 0.5 ml were collected at a flow of 0.5 ml/min, and absorbance was measured at 280 nm. Column was calibrated with high and low molecular weight calibration kit from Pharmacia. The marker proteins were Thyroglobulin Stokes Radius (Rs) 85 Å, molecular weight (Mw) 669 kDa), Ferritin (Rs 61 Å, Mw 440 kDa), Catalase (Rs 52 Å nm, Mw 232 kDa), Aldolase (Rs 48 Å, Mw 158 kDa), Albumin (Rs 35 Å, Mw 67 kDa), Ovalbumin (Rs 30 Å, Mw 43 kDa), Chymotrypsinogen (Rs 20 Å, Mw 25 kDa) and Ribonuclease A (Rs 16 Å, Mw 13.7 kDa). Peak volumes were standardized to a $K_{av} = (V_e - V_o)/(V_t - V_o)$, where V_e is the elution volume of the center of a protein peak, V_o is the void volume (determined by elution of blue dextran), and V_t is the total elution volume.

6. Analytical ultracentrifugation

6.1. Sedimentation velocity

Experiments were carried out at 55,000 rpm and 20 °C in a Beckman Optima-XL-A analytical ultracentrifuge, using 12 mm aluminum double sector centerpieces. The sedimentation coefficient distributions of C30WT and C30ΔL and their respective tagged forms were calculated by C(s) method by direct modelling with distributions of Lamm equation solutions using

SEDFIT program [37]. The mean friction coefficient ratio f/f_0 of the monomer, dimer and trimer were previously determined by Benaroudj et al. [16]. The molecular mass of the different proteins was estimated by combining the experimental Stokes Radius and sedimentation coefficient values using a modified Svedberg equation as described previously [38].

6.2. Sedimentation equilibrium

Experiments were performed with a Beckman Optima-XL-A analytical ultracentrifuge equipped with absorbance optics, using an AN55Ti rotor. Measurements were done at three successive speeds by taking scans at the appropriate wavelength (230 and 235 nm), when sedimentation equilibrium was reached. The equilibrium temperature was 4 °C. High-sedimentation was conducted afterwards for baseline correction. Average molecular speed masses were determined by fitting a sedimentation equilibrium model for a single solute to individual data sets with EQASSOC programs. Data analysis was also performed by global analysis of several data sets obtained at different loading concentrations and speeds using WINNONLIN program [39]. The different species of C30WT and C30WT-His$^+$ were separated by size exclusion chromatography as shown in the *Top* of Fig. 3 and fractions containing the monomer, the dimer, the oligomer and the shoulder were isolated and analyzed separately by sedimentation equilibrium experiments in order to determine their respective molecular weights. For C30ΔL and C30ΔL-His$^+$, the totality of the proteins was analyzed by sedimentation equilibrium. The partial specific volume of C30WT is 0.7263 ml/g at 4 °C, and was calculated from the amino acid composition by SEDNTERP program [40]. The solvent density and the viscosity at 4 °C were calculated with the same program.

7. Results

As shown in Fig. 2, all protein constructs have been expressed in and purified from *E. coli* to near homogeneity and the purity of the proteins was greater than 95%. Self-association properties the C-terminal domain of HSC70 (C30WT: residues 384–646) have been analyzed by size-exclusion chromatography. C30WT

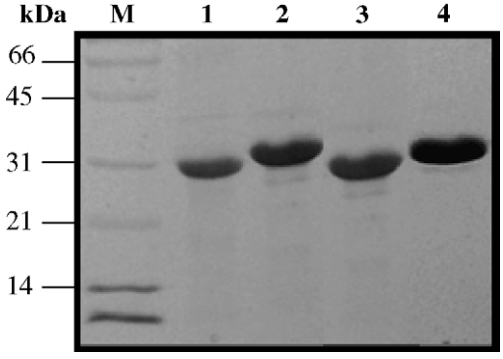

Fig. 2. Analysis of purified proteins by SDS–PAGE. 20 μg of each purified protein were analysed by SDS–PAGE as described under "Section 2". M: molecular mass markers (values in kDa on the left). 15% SDS–PAGE, *line 1*: C30WT; *line 2*: C30WT-His$^+$; *line 3*: C30ΔL; *line 4*: C30ΔL-His$^+$.

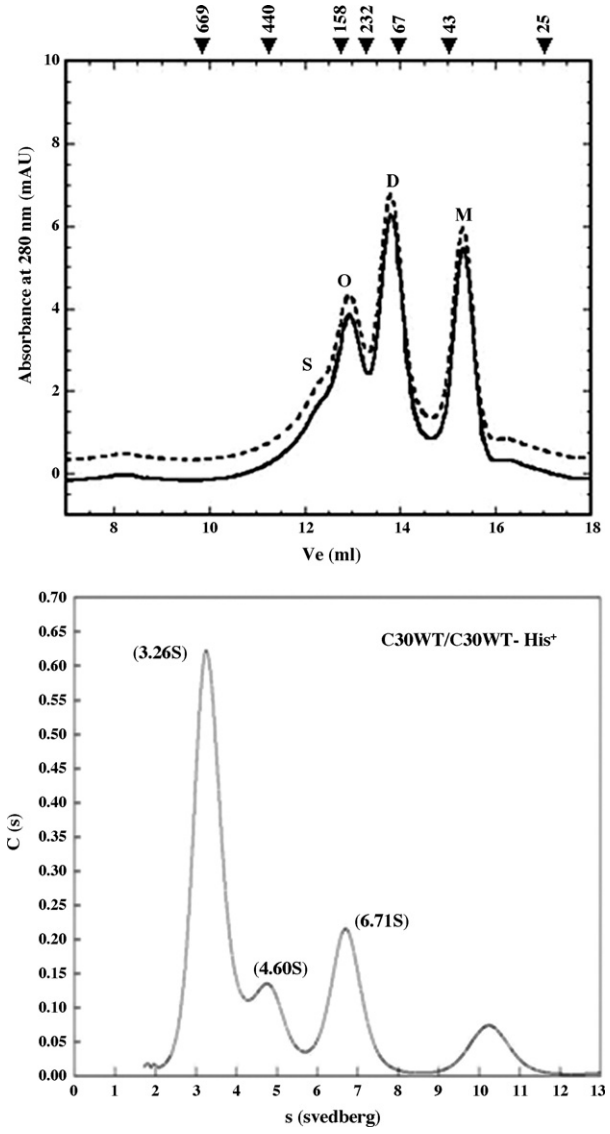

Fig. 3. Structural analysis of C30WT and C30WT-His$^+$. *Top*: analysis of C30WT and C30WT-His$^+$ by size exclusion chromatography, 36 μM of C30WT (*solid line*) and C30WT-His$^+$ (*dotted line*) were loaded on a Superdex200 HR 10/30 column and eluted as described in Section 2. The molecular weight markers used, and shown at the top of panels, are those described under "Section 2". M: monomer; D: dimer; O: oligomer; S: shoulder. *Bottom*: analysis of C30WT and C30WT-His$^+$ by sedimentation velocity, sedimentation coefficient distribution C(s) of 2 mg/ml of each protein at 20 °C from the analysis of the sedimentation profiles at 55,000 rpm.

was also analyzed with the His-tag at its NH_2-terminus (C30WT-His$^+$). As shown in the *Top* of Fig. 3, C30WT and C30WT-His$^+$ both elute after chromatography in three peaks and a shoulder indicating the presence of at least three species with defined Stokes Radii and molecular weights. Similar results are obtained by sedimentation velocity, and at least three sedimentation coefficients could be determined for the two proteins (see the *Bottom* of Fig. 3). The molecular weights of the different species of C30WT and C30WT-His$^+$, observed in their elution profiles (M, D, O and S), were determined by sedimentation equilibrium analysis (data not shown), suggesting that the peak M corresponds to the monomer, the peak D corresponds to the dimer,

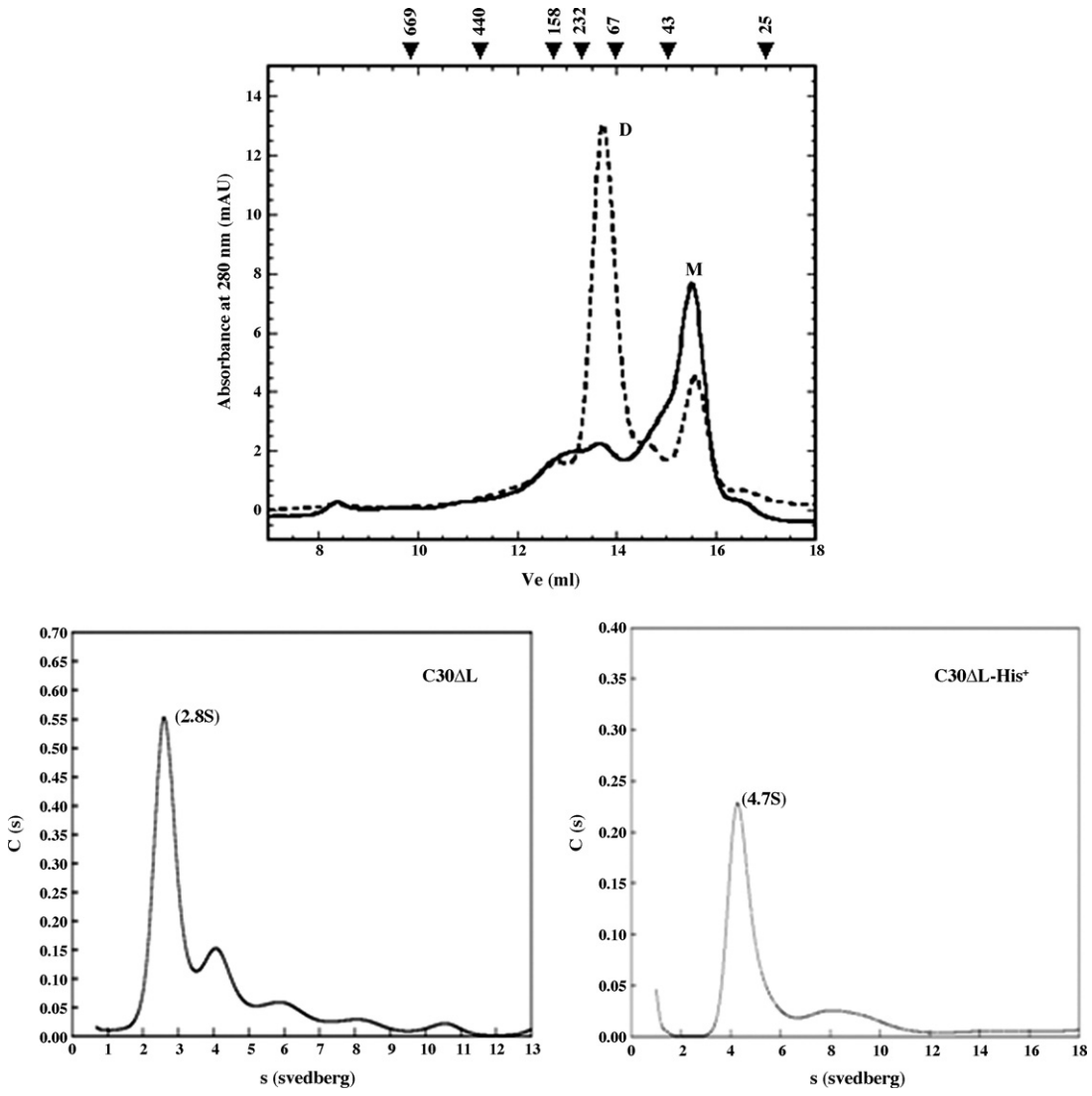

Fig. 4. Structural analysis of C30ΔL and C30ΔL-His$^+$. *Top*: analysis of C30ΔL and C30ΔL-His$^+$ by size exclusion chromatography, 36 μM of C30ΔL (*solid line*) and C30ΔL-His$^+$ (*dotted line*) were loaded on a Superdex200 HR 10/30 column and eluted as described in Section 2. The molecular weight markers used, and shown at the top of panels, are the same than those shown in Fig. 3. M: monomer; D: dimer. *Bottom*: analysis of C30ΔL and C30ΔL-His$^+$ by sedimentation velocity, sedimentation coefficient distribution C(s) of 2 mg/ml of each protein at 20 °C from the analysis of the sedimentation profiles at 55,000 rpm.

the peak O corresponds to the trimer and the shoulder S corresponds to the tetramer.

Self-association properties of the deleted construct C30ΔL (residues 395–646) have been also analyzed in the presence and in the absence of the His-tag, in order to verify if the His-tag has any detrimental effect on the oligomerization of this construct. Results presented in the *Top* of Fig. 4 show that C30ΔL elutes in almost a single peak corresponding to the monomer molecular mass. This result is confirmed by analytical ultracentrifugation indicating that C30ΔL is stabilized into the monomer and gives molecular mass, from sedimentation equilibrium analysis (data not shown), very close to the mass determined from the amino acid sequence of its monomer. The sedimentation coefficient distributions C(s) of C30ΔL and C30ΔL-His$^+$ are presented in the *Bottom* of Fig. 4. These data indicate that the deletion of the four Leucine residues of the interdomain linker, located on the NH_2-terminal end of the compactly folded β-sandwich C-terminal sub-domain, is essential for monomer stabilization and seems to constitute a contact region involved in the oligomerization of HSC70. On the other hand, the self-association properties of the His-tagged C30ΔL (C30ΔL-His$^+$) have been analyzed by size exclusion chromatography and compared to C30ΔL. Fig. 4 shows that C30ΔL-His$^+$ is stabilized in the dimeric form. These results indicate that when the His-tag is placed in the NH_2-terminal end, it promotes the dimerization of the deleted construct C30ΔL.

8. Discussion and conclusion

The use of affinity tags is preferred as purification can be quick, simple, efficient, and can be used at large scale. However, the decision regarding the relative positioning and removing of the affinity tags remains difficult and depends on the primary sequence and conformation of the protein [41]. For recombinant

proteins required for structural/physiological studies or pharmaceutical usage, affinity tags can be removed by chemical reagents, exoproteases, or endoproteases recognizing specific amino acid sequences like enterokinase, thrombin, coagulation factor X_a [42–44]. However, some of the limitations associated with removing tags are: non-specific cleavages generating truncated forms of the protein, addition of one or two extra amino acids, partial removal of the tag, and presence of contaminating proteases in the preparation. In addition, the use of affinity tags for protein purification has some perceived limitations which are: misfolding and/or loss of activity or solubility of the protein, inability to use such fusion proteins for X-ray crystallography or other physical characterization studies, and use of stringent and validated requirements for therapeutic usage [45–48]. Recent reports show that His-tag can alter the binding characteristics or structure of recombinant protein when compared to the wild-type native protein [49,50]. Hang et al. [49] showed that although His-tagged subunits of the terminase enzyme from bacteriophage-λ formed holoenzymes with wild-type catalytic activity, one of the subunits showed altered interaction with DNA. The length, composition and location of His-tag can require further optimization depending upon the sequences of the native protein [50–52]. Nevertheless, there are several studies where affinity tags have no adverse effect on the activity of the native proteins [42,53–59]. Furthermore, introducing an affinity tag may have a positive effect in the biochemical properties of the target protein. A recent literature survey reveals that affinity tags have been observed to: improve protein yield [60], prevent proteolysis [61], facilitate protein refolding and increase solubility [62,63].

In the present study, analysis of the structural basis of self-association of HSC70, using mutant versions, reveals that the deletion of Leu391-Leu394 of the interdomain linker is sufficient to stabilize the protein in the monomeric form, suggesting that this region is involved in self-association. We also report that the hexahistidine-tag, when placed at the NH_2-terminus end of the deleted construct C30ΔL (C30ΔL-His^+), interferes with the oligomerization properties of the protein and stabilizes it in the dimeric form. Although the addition of histidine-tag to the target protein is a simple and well established approach to facilitate purification by Ni^{2+}-agarose column, these results emphasize the need to remove the tag before characterization of some recombinant proteins prior to using them for structural purposes. Additionally, due to the somehow unpredictable changes that adding a tag may introduce in a protein and its behaviour, it is usually desirable to remove it. This reflects on the design of the protein fusion. Importantly, removal of the tag needs to be considered when designing a process for the production of a recombinant protein that is intended for human use to enable production of a "native" protein [64].

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